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REGULAR ARTICLE

Isolation of clonogenic, long-term self renewing embryonic renal stem cells

M. Lusis [a,c], J. Li [a,c], J. Ineson [a,c], M.E. Christensen [b], A. Rice [b], M.H. Little [a,c,*]

[a] *Institute for Molecular Bioscience, University of Queensland, St. Lucia, 4072, Australia*
[b] *Mater Medical Research Institute, Aubigny Place, Raymond Terrace, South Brisbane, 4101, Australia*
[c] *Australian Stem Cell Centre, Australia*

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Abstract A tissue stem cell should exhibit long-term self-renewal, clonogenicity and a capacity to differentiate into the tissue of origin. Such a postnatal renal stem cell has not been formally identified. The metanephric mesenchyme (MM) of the developing kidney gives rise to both the renal interstitium and the nephrons and is regarded as the progenitor population of the developing kidney. However, isolated MM does not self renew and requires immortalization for survival in culture. Here we report the isolation and sustained culture of long-term repopulating, clonal progenitors from the embryonic kidney as free floating nephrospheres. Such cells displayed clonal self renewal for in excess of twenty passages when cultured with bFGF and thrombin, showed broad mesodermal multipotentiality, but retained expression of key renal transcription factors (Wt1, Sall1, Eya1, Six1, Six2, Osr1 and Hoxa11). While these cells did display limited capacity to contribute to developing embryonic kidney explants, nephrospheres did not display *in vitro* renal epithelial capacity. Nephrospheres could be cultured from both Sall1$^+$ and Sall1$^-$ fractions of embryonic kidney, suggesting that they were derived from the MM as a whole and not specifically the MM-derived cap mesenchyme committed to nephron formation. This embryonic renal stem cell population was not able to be isolated from postnatal kidney confirming that while the embryonic MM represents a mulitpotent stem cell population, this does not persist after birth.

Introduction

The kidney is a vital organ in regulating fluid, pH, blood pressure and bone density. The 20th century provided to sufferers of chronic renal disease the possibility of organ transplantation. However, only 15% patients will receive a replacement organ and all face the risk of immunological rejection. As a result of the dramatic increase in cardiovascular disease and diabetes worldwide, the rate of chronic kidney disease (CKD) is estimated to be rising at 7% per annum (Coresh et al., 2007). Hence, there is a strong imperative to seek alternative treatments.

Unlike the liver, resection of a kidney does not elicit organ regrowth. Based on analyses of postnatal cell division, the kidney had been classified as an organ of 'simple duplication', in which repair results from the division of differentiated cells (Messier and Leblond, 1960). The adult brain was traditionally regarded as 'static' implying the absence of both stem cells and proliferation. However, with the isolation of apparently pluripotent cells from a variety of solid organs, including the brain, the possibility that the kidney contains stem cells has

* Corresponding author. NHMRC Principal Research Fellow, Institute for Molecular Bioscience, The University of Queensland, St. Lucia, 4072, Australia. Fax: +61 7 3346 2101.
E-mail address: M.Little@uq.edu.au (M.H. Little).

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been reinvestigated. There have now been many studies suggesting the existence of stem cells in the adult kidney, both within the tubules themselves or in the stroma surrounding these tubules (Hopkins et al., 2009). Using long term BrdU pulse-chase analyses, two different populations of cells have been identified; one in the papilla (Oliver et al., 2004) and the other in the renal tubules themselves (Maeshima et al., 2003, 2006); which appear to display long-term label retention suggestive of a slow cycling stem cell. Other groups have isolated subfractions of the kidney based upon cell surface marker expression (CD24, Sca1, CD133), function (Hoescht efflux) or location (S3 segment of proximal tubule, Bowman's capsule epithelium) (Ronconi et al., 2009; Appel et al., 2009) and demonstrated *in vitro* multipotentiality and/or a capacity to apparently ameliorate acute renal failure *in vivo* (Hopkins et al., 2009). On most occasions, this has been shown to be due to the production of reparative humoral factors rather than cellular integration or transdifferentiation into functional tubular cells (Dekel et al., 2006; Bussolati et al., 2005a; Osafune et al., 2006; Kitamura et al., 2005; Challen et al., 2006). Notably, none of these studies have provided definitive evidence that such endogenous cell populations are clonal, long term self-renewing stem cells. While the ability of the kidney to repair in response to acute injury is substantial, our understanding of how this happens is poor. While attractive to imagine a role for a stem cell here, lineage studies have shown that tubular repair post acute damage does not involve cells from outside of the tubules themselves (Humphreys et al., 2008). Hence, if a postnatal renal stem cell does exist, such a cell must reside in the tubules.

In contrast to our lack of understanding of cellular turnover and potential in the postnatal kidney, within the developing kidney there is a good understanding of the progenitor populations from which this organ is derived. The mammalian kidney arises through interactions between two tissues both derived from the intermediate mesoderm; an epithelial duct called the ureteric bud (UB), which gives rise to the collecting ducts and the ureter, and the metanephric mesenchyme (MM). The MM gives rise to all cell types within nephrons apart from the collecting ducts, as well as giving rise to components of the cortical and medullary renal interstitium, and is regarded as the key progenitor population of the kidney. The development of the nephrons from the MM results from a mesenchyme-to-epithelial transition (MET). During this process, the cells of the MM closest to the tip of the invading UB condense to form the cap mesenchyme (CM). This CM has now been predicted to also represent a stem/ progenitor cell population (Kobayashi et al., 2008). The CM has been predicted to self renew to expand the population in order to generate sufficient cells for nephron induction. All renal tubules aside from the collecting duct are derived directly from CM via MET (Kobayashi et al., 2008). This MET is induced by Wnt9b expression within the ureteric tip (Carroll et al., 2005). In response, the CM on one side of the ureteric tip aggregates and then epithelialises to become a renal vesicle. Sequential MET events occur as the ureteric tree branches such that the last nephrons to arise do so at the periphery of the kidney. Nephrogenesis in the mouse is completed in the first few days after birth (Hartman et al., 2007) and around 38 weeks in the human. While no new nephrons are induced after this point in time, what causes cessation of nephrogenesis and what happens to the CM is not known. One possibility is that the CM is terminally exhausted (Hartman et al., 2007). One would presume, therefore, that at least the component of the MM that gives rise to the CM is also exhausted at this time. The other possibility is that remnant CM/MM populations persist in the postnatal kidney and can be recruited during injury. This has been difficult to investigate as neither CM nor MM has been successfully cultured *in vitro*.

While the challenge in the kidney has been finding a way to demonstrate whether or not a postnatal stem cell exists, the haematopoietic and neural fields have benefited from the development of assays enabling the culture and isolation of stem cells. In the neural field, the neurosphere assay has enabled the cultivation of neural stem cells that show self-renewal capacity (Reynolds and Weiss, 1992). Each sphere represents a clonal population derived from a single stem cell that grows in culture as a light refractive, floating spherical ball. These cells also show the ability to differentiate into all three neural lineages, oligodendrocytes, astrocytes and neurons, as well as cell types from other lineages (Reynolds and Weiss, 1992). The passage of a sphere down to single cell with the indefinite growth of subsequent spheres validates the existence of clonal neural stem cells. While the culture of floating 'spheres' has been attempted in a number of other organs, including heart, mammary gland, dermis and prostate (Messina et al., 2004; Lawson et al., 2007; Toma et al., 2005), in few cases have the criteria of clonality, long term self renewal and retention of potential to develop into the cells of the organ of origin been demonstrated.

In this study, we aimed to answer the question of whether a definitive stem cell population existed within the kidney by establishing the parameters required for nephrosphere culture. To do this, we used embryonic kidney tissue where a known renal progenitor population exists. Here we report the defined media and conditions required to successfully culture nephrospheres from E12.5 to E17.5 embryonic kidneys. These cells display broad mesodermal potential and retain the expression a number of key developmental markers of the MM. However, such spheres substantively lose the capacity to epithelialise, displaying instead the broader mesodermal potential of the intermediate mesoderm. Of importance, nephrospheres could not be isolated from postnatal kidney. This supports the hypothesis that the embryonic stem/ progenitor cell population of the kidney is lost after the cessation of nephrogenesis. It also implies that any true stem cell population existing within the postnatal kidney does not represent this embryonic progenitor population.

Methods and materials

Nephrosphere Culture

Embryonic kidneys were micro-dissected from CD1 or C57BL/6 mouse strains, minced and dissociated using Accutase (Chemicon), 37 °C, for 7 (E11.5-E13.5) to 12 minutes (E15.5-E17.5). After dissociation, preheated DMEM:F12 (Gibco) containing BSA (0.05% weight/volume, Sigma) and Dnase (10 μg/ml, Roche) was added to the digestion solution and centrifuged at 1,500 rpm, 3 min (Rotofix, Hettich centrifuge). Cells were resuspended in 0.5 ml nephrosphere media (see below), titurated to single cells and plated (T25 flask (Nunc)) at 2.5×10^5 cells/ ml. Subsequent passaging of spheres was performed using digestion at 37 °C, 3 min and replating at

1.25×10^5 cells/ ml. Conditioned media collected from the previous passage was syringe-filtered (0.22 μm filter) and added at a ratio of 1:2.85 sphere media (the first primary cell culture did not use conditioned media). GFP-labelled nephrospheres were derived from embryos of crosses between B5/GFP (Hadjantonakis et al., 1998) males x wildtype CD1 females. Adult kidney was finely minced and digested with collagenase B (1 mg/ml), dispase II (1.2U/ml) in HANKS balanced salt solution (Sigma), 37 °C, 10 min. Digestion was stopped by adding 100% fetal calf serum (FCS) and single cells in suspension collected using centrifugation. Two further rounds of digestion and harvesting of occurred before pooled supernatants were filtered through sequential cell strainers (70 um and 40 um) before centrifugation at 300rcf, 5 mins, 4 °C. The pellet was resuspended in nephrosphere media and seeded as described above. All cell culture curves are presented as cumulative cell totals.

For derivation of nephrospheres from Sall1-GFP transgenic mice (Osafune et al., 2006; Takasato et al., 2004), kidneys were isolated, dissociated as described above and filtered through sequential 70 μm and 40 μm strainers. Isolated cells were then sorted into GFP-positive and GFP-negative fractions using FACS Aria (BD Bioscience) excluding dead cells. The resulting fractions were cultured as nephrospheres as described.

Nephrosphere Media

Basal nephrosphere media contained low glucose DMEM (1 g/L, Gibco) and F-12 (Gibco) (1:1); β-mercaptoethanol (0.25 mM, Sigma); HEPES (15 mM, Thermo Corporation); insulin-transferrin-sodium selenite media supplement (ITS) (insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), Sigma); Glutamax (2 mM, Invitrogen); B27 proliferation supplement (5x concentration, Gibco); Penicillin/Streptomycin (10 U/ml); bovine recombinant bFGF (20 ng/ml, Roche); heparin (0.7 U/ml, Sigma); and thrombin purified from bovine plasma (2 U/ml, Sigma). Growth factors added to the basal media included purified EGF (20 ng/ml, BD Biosciences); recombinant mouse IGF-1 (20 ng/ml, Biosource); recombinant human HGF (20 ng/ml, R&D Systems).

Bone Marrow-Derived Mesenchymal Stem Cells

Bone marrow derived mesenchymal stem cells (BM-MSC) used for comparative gene expression analyses were obtained from the Mater Medical Research Institute (gift of Kerry Atkinson and Gary Brooke) as previously described (Short et al., 2009). In brief, wild-type C57BL/6 mice were sacrificed at 8-12 weeks of age. For isolation of bmMSC, the pelvis, tibias and femurs were removed from the mice, cleaned of excess tissue and transferred to ice cold αMEM (Invitrogen, Carlsbad, CA). Bones were thoroughly crushed using a mortar and pestle, digested in Type I collagenase (100 U/ml; Worthington Biosciences, Lakewood, NJ) and DNase I (10 μg/ml, Roche, Basel Switzerland), 37 °C for 20 min, filtered through a 70 μm filter before resuspension in phosphate buffered saline (PBS) and 62.5% Percoll (GE Healthcare, Rydalmere, Australia). Cells were centrifuged at 400 *g* (1600 rpm) for 20 min, the interface collected, resuspended in αMEM + 20% FCS and incubated at 37 °C, 5% CO_2 After 72 h, non-adherent cells were removed and adherent MSCs retained. All procedures were approved by the University of Queensland Animal Ethics Committee.

RT-PCR

RNA was isolated from murine nephrospheres, bone marrow-derived murine mesenchymal stem cells and murine E13.5 embryonic kidneys using RNeasy Mini Kit (Qiagen) including on-column DNase digestion (Qiagen) according to manufacturer's instructions. RNA concentration and quality was determined using a spectrophotometer (Nanodrop). cDNA was prepared using Superscript II RNase H- Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions, using 500 ng of RNA per reaction. Primer sequences for all genes analysed are reported in Supplementary Data 1.

Immunofluorescence of spheres

To conduct immunofluorescence on undissociated spheres, glass coverslips were coated with Cell-Tak (BD Biosciences) as per the manufacturer's instructions. Spheres or single cells were fixed in 4% paraformaldehyde, 30 minutes, made permeable with 0.2% Triton X-100, 10 minutes, and blocked for 15 minutes in 1% BSA in PBS. Primary antibodies diluted in 1% BSA in PBS were incubated overnight at 4 °C. Secondary antibodies diluted in 1% BSA in PBS were incubated at room temperature, 60 minutes. DAPI (1 μg/ml; Boehringer Mannheim) was added for the last 5 minutes of incubation. Cells were subsequently washed 3×5 minutes in PBS and mounted with Vectashield (Vector Laboratories) and photographed.

Multipotentiality and tubulogenic assays

For adipogenesis and osteogenesis assays, cells were plated at 1.5×10^5 cells/ml in a 6 well plate and incubated until confluent in DMEM supplemented with 10% FCS, ITS supplement (as above), Glutamax (2 mM), penicillin/streptomycin (10 U/ml)). Upon confluence, media was changed to differentiation media and cells were cultivated for an additional three weeks.

Differentiation media for adipocytes: DMEM (Gibco); 10% FCS; insulin (5 μg/ml); dexamethasone (1 μM); 3-isobutyl-1-methylxanthine (IBMX) (500 μM); and penicillin / streptomycin (10 U/ml). Adipocytes were stained with Oil-Red-O (Molecular Probes) in PBS (1:2000) for 10 minutes.

For osteocyte differentiation, cells were cultured in DMEM (Gibco); ascorbic acid (0.2 mM); B-glycerophosphate (10 mM); dexamethasone (100 nM); and penicillin/streptomycin (10 U/ml). Cells were washed in PBS, fixed for 10 minutes in 4% paraformaldehyde in PBS and then finally washed in PBS. Osteocytes were stained for alkaline phosphatase activity using 0.1 mg/ml Fast Blue BB salt and Napthol AS-MX phosphatase in 0.1 M Tris-HCl (pH8.5), 2 mM $MgCl_2$ for 20 minutes. Alizarin Red S solution was made using 1 g in 100 ml H_2O and pH adjusted to 4.1-4.3 using 0.5% ammonium hydroxide and stained for 20 minutes.

For chondrocyte differentiation, 2.5×10^5 cells were centrifuged at 1500 rpm for 5 minutes in a 15 ml Falcon tube. 300 μl of differentiation media was added containing DMEM, dexamethasone (100nM), ascorbic acid (0.2 mM), ITS supplement (as above), TGFβ3 (10 ng/ml, R&D Systems),

penicillin/streptomycin (10 U/ml). Media was changed twice a week for three weeks. The pellet was fixed in 4% paraformaldehyde in PBS, processed, embedded in paraffin and sectioned. The sections were taken through xylene and rehydration steps as for reaggregations (described below). Pellet was stained for 20 minutes in Alcian Blue using 1 g of Alcian Blue powder dissolved in 100 ml of 3% acetic acid and adjusted to pH 2.5 with acetic acid. Sections were washed in tap water for 2 minutes and then counterstained with nuclear fast red solution for 5 minutes (Vector Laboratories) before dehydration. For myogenic differentiation, 5×10^4 C2C12 cells (mouse myoblast cell line) were seeded with 5×10^4 GFP labelled cells from nephrospheres and allowed to grow to confluence in a six well plate format containing 2mls per well of DMEM, 10% FCS, ITS supplement (same concentration as above) and penicillin / streptomycin (10 U/ml). Upon confluence, media was changed to low serum media comprised of DMEM, 1% horse serum, 1% serum supreme (BioWhittaker) and penicillin / streptomycin (10 U/ml) (Di Rocco et al., 2006). Cells were allowed to differentiate for the following two weeks and photographed.

For Matrigel assays, growth factor reduced Matrigel (BD Bioscience) was defrosted overnight at 4 °C, 150 μl of Matrigel was mixed with 150 μl of ice cold media containing either whole or dissociated nephrospheres, and the combined 300 μl plated per well in a 24 well plate. The ice cold media comprised DMEM (1 g/L glucose), F12 (1:1), ITS supplement (same concentration as above), glutamax (2 mM), penicillin / streptomycin (10 U/ml), and growth factors with final concentration as follows: EGF (25 ng/ml, BD Bioscience), bFGF (20 ng/ml Roche), and HGF (40 ng/ml, R&D Systems).

Assessment of potential to undergo epithelialisation in response to Wnt4 was determined by culturing sphere cells on a lawn of NIH3T3 cells pre-treated with mitomycin, overexpressing Wnt4, as previously described (Osafune et al., 2006). The outcome was assessed using brightfield microscopy and immunofluorescence for E-cadherin and Wt1.

Immunophenotyping

Approximately 1×10^5 nephrosphere cells or MSCs were incubated in 100 μl with fluorescein isothiocyanate (FITC), phycoerythrin (PE) conjugated or unconjugated primary antibodies for 30 minutes at 4 °C. The following directly conjugated anti-mouse monoclonal antibodies (BD Pharmingen) were used: CD45-FITC (clone 30-F11); Sca-1-PE (clone E13-161.7); CD31-PE (clone MEC 13.3); CD44-APC (clone IM7). Non conjugated goat anti-mouse CD34 (clone RAM34) and CD29 (clone KMI6) (BD Pharmingen) were used in combination with the appropriate FITC conjugated secondary (Sigma). Appropriately conjugated isotype controls were used to determine non-specific binding (BD Pharmingen). Finally, 2 μg/ml 7-aminoactinomycin D (7-AAD, Sigma) was added to each sample to exclude non-viable cells. All FACS data is representative of at least three individual experiments. Cells were analysed with a BD LSR II Analyser.

Mixed Lymphocyte Reaction

Suppression of T cell proliferation by bone marrow-derived MSC or nephrospheres isolated from either C57BL/6 or CD1 mouse strains was measured using MLR. These cultures were carried out in U-bottom, 96-well plates (Greiner) in a total volume of 200 μl of complete media consisting of αMEM supplemented with 10% FCS and 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-gluatamine, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; all Invitrogen) and 50 μM 2-mercaptoethanol (Sigma, MO, USA). BALB/c [H-2^d] stimulator splenocytes cells, isolated by density centrifugation (Lympholyte-M; Cedarlane, Canada), were cultured overnight with 100 ng/ml LPS in culture media in a humidified 37 °C, 5% CO_2 incubator, then irradiated (2000 cGy) prior to co-culture (Turner et al., 2008). MSC (passage 8; passage number did not effect immunosuppressive capacity of MSC [data not shown]; 3000 cells/well) or nephospheres (passage 4; 3000 cells/well) were allowed to adhere overnight before being irradiated (3000 cGy) prior to co-culture. Responder T-cells derived from C57BL/6 [H-2^b] or B10.Br [H-2^k] mice were purified using a pan T-cell isolation kit (Miltenyi Biotec, Gladbach Germany). Stimulator cells (2×10^5 cells/well) and responder T-cells (3×10^5 cells/well) were co-cultured ± MSC or nephrospheres in a humidified 37 °C, 5% CO_2 incubator. Proliferation was assessed by [^{3}H]-Thymidine incorporation (1 μcurie/well) (Amersham Biosciences, U.K.) after a total of 96 hours of culture. Cells were harvested using the TOMTEC 96-well Mach III Harvester (Perkin-Elmer, Vic, Australia) and counts per minute (cpm) measured on a 1450 MICROBETA TRILUX β-scintillation counter (Perkin-Elmer, Vic, Australia). The data represents the average of 9 technical replicates from one biological sample. Statistics were calculated using one way analysis of variance with post hoc Dunnett's test (comparing all samples to controls).

Recombination assay of ex vivo embryonic renal potential

Twenty four hours prior to conducting the assay, NIH3T3 cells overexpressing Wnt4 (gift of Andreas Kispert) were seeded on polycarbonate filters (0.4 μm pore size) at a density of 5×10^4 cells in 50 μL volume. Polycarbonate filters (0.4 μm pore size) were coated with mouse collagen IV solution (100 μg/ml, BD Biosciences) for one hour prior to washing with PBS. These filters were placed on top of the feeder cells, with both filters being supported by a metal grid in a centre well organ culture dish (Falcon). Media comprised DMEM (Gibco), 10% FCS, ITS supplement (same concentration as above) and Glutamax (2 mM). E12.5 kidneys were dissociated to single cells as per the nephrosphere. Embryonic kidney cells and GFP$^+$ nephrosphere cells were mixed together, centrifuged at 2,000 rpm for 2 minutes and the entire pellet placed on top of the collagen IV coated filter and allowed to develop for four days. The pellet was fixed in 4% paraformaldehyde in PBS and subsequently processed prior to paraffin embedding and sectioning. Slides were deparaffinised and rehydrated through and ethanol series. Antigen unmasking solution (Vector Laboratories) was used with slides boiled in a microwave for 12 minutes and then allowed to cool on the bench for around 60 minutes. Slides were washed 3×2 minutes in PBS and then blocked in 2% sheep serum for 60 minutes at room temperature. Primary antibodies, diluted in 2% sheep serum/ PBS, were incubated

overnight at 4 °C while secondary antibodies were incubated in the dark at room temperature, 60 minutes. Primary antibodies are detailed in Supplementary Data 2.

Culture under renal capsule

C57/BL6 mice were anesthetised using 2% isofluorane in oxygen and approximately 5×10^5 cells from dissociated nephrospheres (derived from CD1 embryonic kidneys) in 50 μL volume were injected under the renal capsule. After three weeks, kidneys were fixed in 4% paraformaldehyde, processed, paraffin embedded and sectioned. Histology was assessed using haematoxylin and eosin or Masson's trichrome staining.

Results

Developing a nephrosphere assay

In order to identify kidney stem cells based on sphere formation, we used embryonic day (E) 12.5 mouse kidneys (Fig. 1A) dissociated to single cells. Using a serum free basal media (Wachs et al., 2003), combinations of growth factors that had either been used in successful adaptations of the neurosphere assay in other organs, or that were noted to be important in renal development were assessed. These included bFGF; EGF; thrombin; LIF; TGFβ2; TGFalpha; HGF; BMP7; IGF1; GDNF; Activin A and thyroid hormone. The requirement for supplements including ITS, dexamethasone, B27 supplement, N2 supplement; high and low glucose DMEM, and β-mercaptoethanol, were also assessed. Cell cultures were observed for proliferation, the presence of any spheres or, at minimum, free floating phase bright cells versus an adherent fibroblastic morphology. Dissociated embryonic kidney cultivated in previously described conditions for neurosphere culture (Reynolds and Weiss, 1992) did not form spheres, instead forming an adherent monolayer. In contrast, formation of spheres (referred to as nephrospheres) of up to 100 μm in diameter were observed in the presence serum-free basal media (Wachs et al., 2003) with the addition of thrombin and bFGF (Fig. 1B). The nephrospheres showed a similar morphology to neurospheres, being semi-adherent or floating, with phase bright appearance (Fig. 1Ba). Nephrospheres were solid balls of cells in which the component cells were surrounded by significant amounts of extracellular matrix (Fig. 1Bb). The nuclei of cells within nephrospheres appeared large and poorly stained for Toluidine Blue, suggestive of a relative absence of condensed chromatin. This is similar to embryonic stem cells.

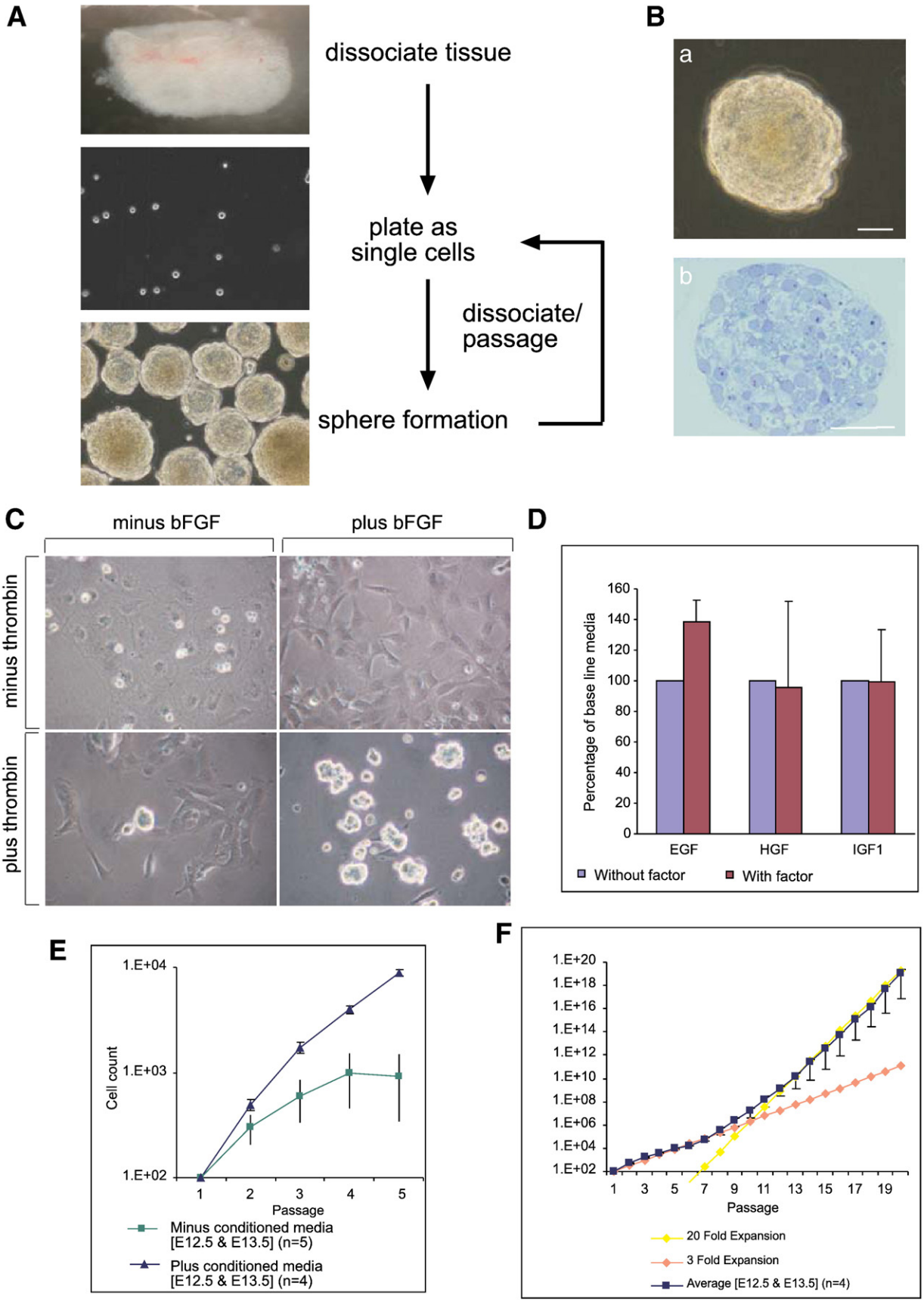
The impact of bFGF and thrombin on nephrosphere formation was assessed by seeding cells from dissociated E12.5 murine kidneys in media with and without each factor (Fig. 1C). Without bFGF or thrombin, there was limited proliferation and a high proportion of cells were unviable as assessed using trypan blue staining. Addition of bFGF facilitated proliferation, however the cells became adherent and grew as a monolayer. Limited proliferation was observed with the addition of thrombin alone, although this did reduce cell adherence. With the addition of both thrombin and bFGF, the majority of cells remaining phase bright and sphere formation occurred (Fig. 1C). No other combination of media or growth factors facilitated the derivation of nephrospheres. The impact of the addition of IGF-1, HGF and EGF to the basal media/ bFGF/ thrombin combination was then reassessed (Fig. 1D). Addition of HGF and IGF1 did not appear to make any difference to sphere formation. Addition of EGF did provide a higher yield of cells from spheres at the first passage, however this incremental yield did not continue over subsequent passages (data not shown). While nephrosphere media provided reproducible sphere formation, there was a decrease in proliferation over time (Fig. 1E). The addition of conditioned media from the previous passage of spheres addressed this decline in expansion and enabled long term culture (Fig. 1E). The use of conditioned media has previously been used in some neurosphere and skin derived precursor assay protocols both to establish and passage the cells (Wachs et al., 2003; Bearzi et al., 2007).

Nephrospheres displayed a characteristic 'S' shaped proliferation curve (Fig. 1F). During early passages (passage 1-8), spheres displayed a 2-3 fold increase in cell number between passages (Fig. 1F). There was a lag in proliferation around passage 5, after which proliferation would increase. This lag stage corresponds with the period where, without conditioned media, the cells would reach senescence. During later passages (passage >10), sphere cells showed a 20 fold increase in cell number between passages. Between passage 3 and passage 13, the number of population doublings per day increased from 0.3 +/- 0.03 SEM to 0.6 +/- 0.12 SEM.

To determine whether nephrospheres showed clonality, dissociated spheres were seeded at two cells per well in 96 well plates. An average of 2.2% (+/- 1.1% SEM) of cells formed spheres (defined as spheres with a diameter in excess of 50 μm). When cells were seeded in methyl cellulose, the clonal sphere forming efficiency was approximately 2%, consistent with liquid culture results. This is comparable with the rate of clonality seen in neurospheres, where 2.4% of cells dissociated from neurospheres are capable of generating a secondary neurosphere (Reynolds and Rietze, 2005). When cultured at high density, nephrospheres formed due to aggregation rather than clonal growth, as previously reported for neurospheres (Jessberger et al., 2007; Singec et al., 2006). This was established by co-culturing GFP^+ and unlabelled sphere-derived cells and observing mixed colonies. A full description of the process of nephrosphere initiation and early growth characteristics is provided in Supplementary Data 3.

Nephrosphere gene expression and immunophenotype

In order to characterise the gene expression of nephrospheres, key genes marking the MM, CM and other embryonic renal sub-compartments were examined using RT-PCR. Nephrosphere cultures derived from E12.5 embryonic kidney at passages four, eight and twelve were examined to determine whether the expression of analysed genes changed across time in culture (Fig. 2A). The experiment was conducted using three pooled samples of RNA per time point from separate nephrosphere cell lines. Murine bone marrow-derived MSC (BM-MSC) were also examined as an example of a distinct somatic stem cell population. All PCR primers were designed to span introns to ensure a unique


A
dissociate tissue
plate as single cells
dissociate/ passage
sphere formation
B
a
b
C
minus bFGF
plus bFGF
minus thrombin
plus thrombin
D
Percentage of base line media
160
140
120
100
80
60
40
20
0
EGF
HGF
IGF1
Without factor
With factor
E
Cell count
1.E+04
1.E+03
1.E+02
1
2
3
4
5
Passage
Minus conditioned media [E12.5 & E13.5] (n=5)
Plus conditioned media [E12.5 & E13.5] (n=4)
F
1.E+20
1.E+18
1.E+16
1.E+14
1.E+12
1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1
3
5
7
9
11
13
15
17
19
Passage
20 Fold Expansion
3 Fold Expansion
Average [E12.5 & E13.5] (n=4)

RNA-derived amplicon (Supplementary Data 1). Gene expression in nephrosphere cultures across time was relatively stable. Nephrospheres were seen to express known markers of both MM and CM (Wt1, Osr1, Hoxa11, Eya1, Six1, Six2, Sall1, Gdnf). In addition, the CM gene cadherin 11 was also expressed. Expression of Eya1 and Osr1 was initially low and appeared to increase with time. This may reflect the selection of a subpopulation of cells over time in culture or the gradual increase in expression of these genes in the population as a whole. Of note, Osr1 is also expressed in the intermediate mesoderm. Transcription factors marking the nephric duct (Lhx1) and UB (Pax2) were not expressed. Expression of Pax2 also occurs in the CM, but neither Pax2 nor Pax8 were expressed in the nephrospheres. The renal stromal marker Foxd1 was also not detectable by RT-PCR until the latest passage. Nephrospheres did not express megalin, a marker of differentiated tubular epithelium in the developing kidney. The endothelial progenitor marker Flk1 was, expressed as was Sca-1, a commonly stem cell antigen, and PDGFBB, which in the kidney is normally expressed on endothelium.

Immunofluorescence was also used to investigate nephrosphere phenotype (Fig. 2B). Nuclear WT1 protein was observed in most cells of the intact nephrosphere as well as cells dissociated from these spheres and cultured in 10% FCS for 24 hours. This protein is expressed in MM and CM. The intermediate filament protein, nestin, which is expressed in the peripheral MM and the podocytes of the developing kidney and is upregulated within the interstitial fibrotic regions of chronically damaged kidneys, was only heterogeneously expressed within nephrospheres (Fig. 2B). Nestin, also regarded as a marker of BM-MSC, has been variably reported in neurospheres (Campos et al., 2004; Gritti et al., 1996) and is upregulated in Wilms' tumour (Murphy et al., 2009).

Similarities between MSCs and nephrospheres

Surprisingly, a number of genes frequently used as markers of developing kidney (Eya1, Six1, Six2, Osr1, cadherin 11 and GDNF) were also expressed in BM-MSC. BM-MSC also expressed markers of renal vesicle and proximal tubule (Wnt4, megalin) and the stromal marker Foxd1, but did not express the MM markers Wt1, Sall1 or Pax2. This overlap of gene expression raised speculation as to potential lineage relationships between MSC and nephrospheres. MSC from all locations are characterised principally by their mesodermal differentiation potential, as well as their expression of certain cell surface molecules (Dominici et al., 2006). By definition, MSC grow as adherent fibroblastic colonies. While originally isolated from the bone marrow, MSC have now been isolated from many fetal and postnatal tissues, including postnatal fat, placenta, amnion and kidney (Meirelles et al., 2006). Hence, we assessed the expression of selected cell surface molecules using immunophenotyping (Fig. 2C). Nephrospheres were $CD34^{-}CD45^{-}CD44^{+}CD29^{+}Sca1^{lo}$, consistent with that previously reported for murine MSC (Meirelles et al., 2006). However, such surface proteins are in no way restricted to MSC populations.

MSC are functionally characterised as possessing immunosuppressive activity. This is often assessed by their capacity to suppress proliferation of T cells using a mixed lymphocyte reaction (MLR) (Bartholomew et al., 2002). Recently, such immunosuppressive characteristics have been shown to not be restricted to MSC and may represent a general property of fibroblastic/stromal cells (Haniffa et al., 2007). Nephrosphere cells from either a C57BL/6 or outbred CD1 background were assessed for the immunosuppressive capacity compared to BM-derived C57BL/6 MSC. Consistent with previous studies, C57BL/6 MSC demonstrated strong immunosuppressive capacity when MHC matched to the responder cells (C57BL/6 Responders and stimulators: Mean - 5056±697 CPM; co-cultured with MSC: Mean - 1573 ±262 CPM, $p<0.0001$). This was also true when the MSC were MHC mismatched to the responders (B10.Br Responders and stimulators: Mean - 6625±805 CPM; co-cultured with MSC: Mean - 1095±200 CPM, $p<0.0001$). C57BL/6 nephrospheres showed a weak but statistically significant reduction ($p<0.05$) in T cell proliferation compared to when no nephrospheres were added (C57BL/6 responders and nephrospheres – mean: 4227±808 CPM; B10.Br responders and nephrospheres – mean: 5522±608CPM). There was no reduction in T cell proliferation seen in either setting when nephrospheres from a CD1 background were co-cultured with the responders and stimulators (Fig. 2D). We conclude that nephrospheres have limited immunosuppressive capacity in comparison to BM-MSC or that only a subpopulation of nephrospheres displays this property.

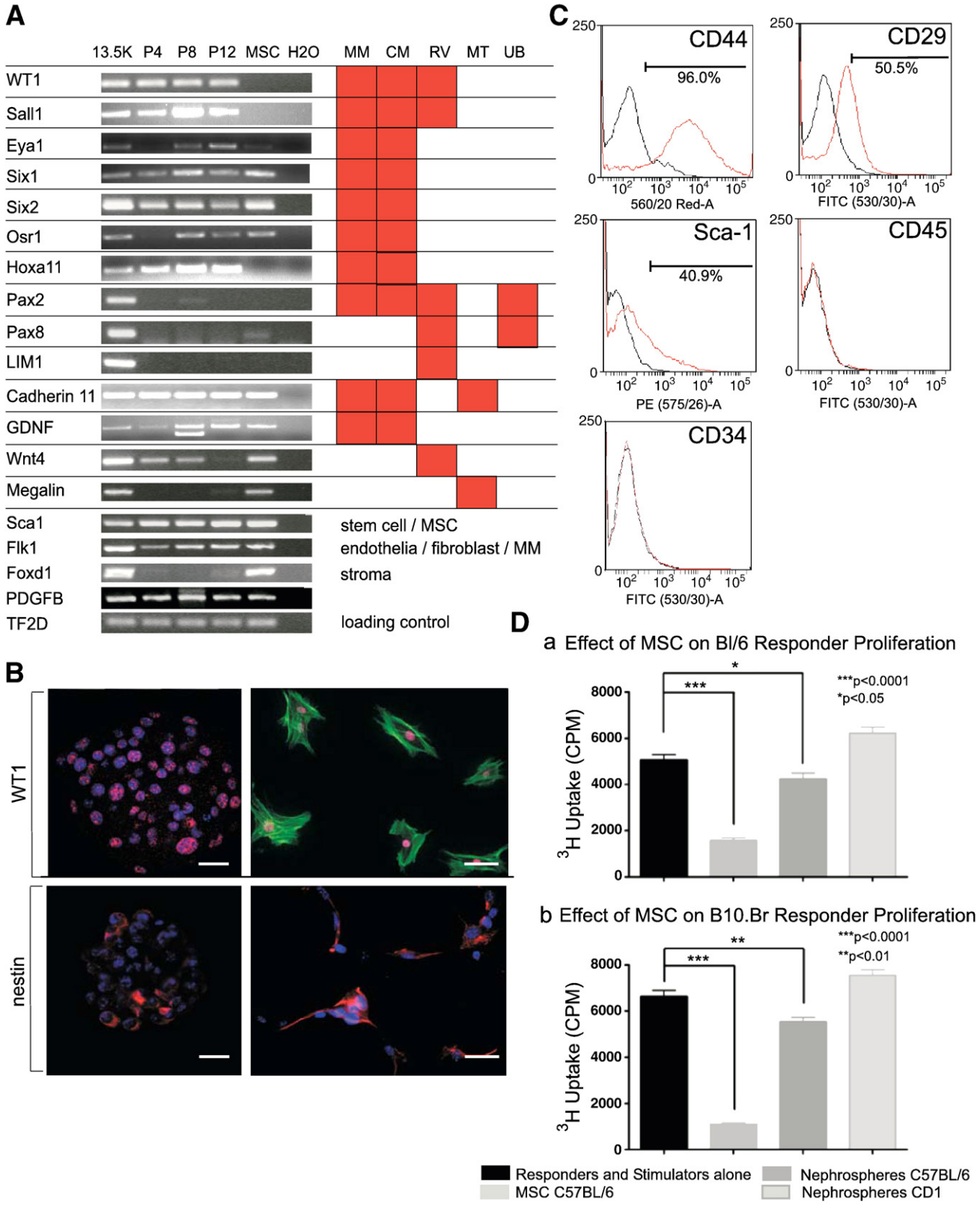
Nephrospheres show broad mesodermal multipotentiality in vitro and in vivo

The mesodermal multipotentiality of nephrospheres was assessed *in vitro* using standard protocols for the mesodermal differentiation of MSC. Nephrosphere-derived cells showed adipocytic, chondrogenic and osteogenic potential.

Figure 1 Isolation of nephrospheres and determination of optimal growth parameters. A) Strategy for isolation of nephrospheres from embryonic kidney. E12.5 kidneys (top panel) were dissociated to single cells and plated in sphere media (middle panel), resulting spheres (nottom panel) were serially disaggregated for replating and subsequent sphere culture; B) Morphology of resulting colonies; a) brightfield morphology of a mature nephrosphere; b) thin section of nephrosphere; scale bars: 50 μm; C) Analysis of the effect of addition of conditioned media on the longevity of self renewal; D) Representative images of dissociated embryonic kidney cultures after 6 days in culture demonstrating the effect of thrombin and bFGF on morphology. Nephrospheres only arose in the presence of both thrombin and bFGF (bottom right); scale bars:50 μm; E) Analysis of the effect on proliferation of addition of IGF1, HGF and EGF to nephrosphere media; F) Average growth curve of nephrospheres isolated from E12.5 or E13.5 embryonic kidneys for 20 passages. Nephrosphere cultures displayed an average expansion of around 3 fold per passage for the first ten passages. After around passage 10, the proliferation rate increases, with approximately 20 fold expansion between passages. Y-axis represents cumulative increase in cell numbers. The starting cell count of 100 at passage 1 is an arbitrary figure.

They were also able to form GFP$^+$ myotubes when co-cultured with C2C12 cells (see Figs. 3A-H). These studies were performed using both early and late stage nephrosphere cultures, with results being consistent at all passages.

Mesodermal potential was also assessed in an *in vivo* environment by injecting 5×10^5 nephrosphere cells under the renal capsule, a location where porcine embryonic kidney explants display broad non-renal potential (Dekel et



al., 2006; Robertson, 2007). A variety of cell morphologies were observed, including adipocytes, Alcian Blue-positive tissue (cartilage) and areas in which Masson's trichrome staining indicated the presence of collagen deposition (Figs. 3I-L). No evidence of epithelial differentiation or the development of tubules was visible. Control experiments injecting PBS alone showed no cellular growth under the capsule. In conclusion, nephrospheres demonstrate broad mesenchymal potential *in vitro*, similar to MSC.

Assessing the capacity of nephrospheres to epithelialise and/or form renal tubules

Matrigel has been used as a three dimensional scaffold to provide an environment for *in vitro* tubulogenesis and angiogenesis (Dontu et al., 2003; Santos et al., 1993). Dissociated postnatal mouse kidneys or cultured adult rat and rabbit proximal tubular cultures placed into Matrigel form tubules in response to growth factors (Taub et al., 1990; Bowes et al., 1999; Han et al., 2004). Nephrospheres were either seeded as single cells or as un-dissociated spheres in Matrigel supplemented with EGF, bFGF and HGF. Extensive processes formed generating apparently multicellular elongated aggregates. Confocal immunofluorescence and thin section analysis showed that these structures did not possess a lumen and displayed morphology more similar to endothelia or myotubes (Fig. 3M-P). Renal epithelial potential was assessed by placing nephrosphere cells in contact with NIH3T3 cells stably producing Wnt4 (Osafune et al., 2006). Freshly isolated Sall1-positive cap mesenchyme is known to be able to form epithelial colonies in this environment, however, no epithelial progenitor colonies formed from nephrospheres under these conditions.

Assessing the renal potential of nephrospheres

While nephrosphere cells failed to form epithelial colonies, to test whether they retained a capacity to form nephrons when placed in an appropriate environment, we developed a novel renal recombination assay by adapting protocols previously described by Osafune et al. (2006) and Kispert et al. (1998) (Fig. 4Aa). Here, whole E12.5 kidney dissociated to single cells was mixed with cells dissociated from nephrospheres, centrifuged into a pellet and deposited on collagen IV-coated filters placed above a Wnt4 over-expressing feeder layer for 4 days (Fig. 4A). Such chimeric kidneys underwent tubulogenesis within two days (Fig. 4A). In order to track the nephrosphere-derived cells, spheres were derived from embryonic kidney isolated from a constitutively GFP-labelled mouse (Fig. 4A). While most nephrosphere-derived cells were detected in the interstitium between the epithelial structures, on occasion nephrosphere-derived cells were able to engraft into epithelial structures including nephron tubules (Fig. 4B), suggesting that at least a subpopulation of these cells has epithelial potential. However, GFP-positive cells were never observed in a CM location surrounding a calbindin-positive ureteric epithelial vesicle, implying that this may not represent a conventional MET as observed during normal development.

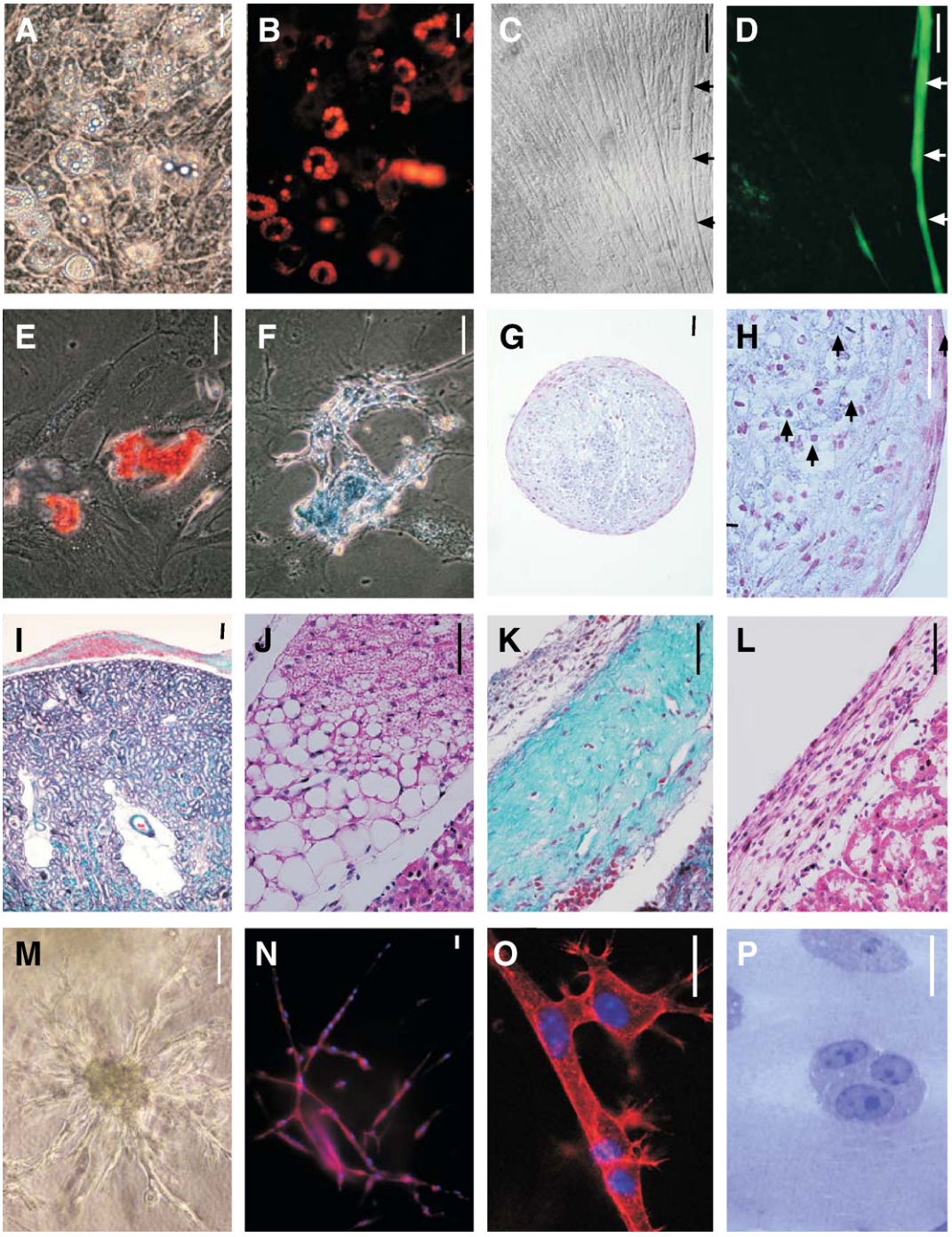
Nephrospheres are derived from the metanephric mesenchyme and are not present in the postnatal kidney

To examine the temporal presence of nephrosphere-forming cells, nephrospheres were isolated from embryonic kidney collected between E11.5 and E17.5. A significant difference was observed between early and late stage kidneys in both the ease of establishment and their proliferative capacity over time. With embryonic kidneys older than E15.5, there was a limited increase in cell yield over the first five passages (Fig. 5A). More adherance was observed in cultures derived from later stage embryonic kidneys, however long term repopulating spheres were able to be isolated. When tissue was isolated from adult kidney (>postnatal day 30), a variety of cell morphologies were observed, including single phase bright adherent cells and fibroblastic populations (Fig. 5B). In addition, occasional tissue aggregates resembling 'spheres' initially formed (Fig. 5Ba), but these were never able to be cultured beyond the first passage. Hence initial 'sphere' formation does not indicate long term potential.

Figure 2 Gene expression pattern, immunophenotype and immunosuppressive capacity of nephrosphere cultures. A) Reverse transcriptase polymerase chain reaction analysis of the expression of a panel of kidney development and stem cell genes in passage 4, 8 and 12 nephrospheres isolated from E12.5 embryonic kidney. These can be compared to gene expression in the E13.5 total embryonic kidney and BM-MSC. The right panel indicates the anticipated gene expression pattern for each gene during kidney development. B) Immunofluorescence for WT1 or nestin on whole (left) and disaggregated (right) nephrospheres. In both cases, cells were stained for either Wt1 or nestin (red) along with phalloidin (green). Scale bars represent 20 μm for whole spheres and 50 μm for adhered cells. C) FACS analysis of nephrospheres for CD44, CD29, Sca-1, CD45 and CD34. D) Mixed lymphocyte reactions to assess the ability of nephrospheres to suppress proliferation of T cells activated in response to BALB/c [H-2^d] stimulator cells. a) C57/Bl6 (responders) [H-2^b] – representing a full MHC mismatch to the stimulators but **not** the Bl/6 background MSC or nephrospheres ("donor derived" MLR); b) B10.Br (responders) [H-2^k], representing a full MHC mismatch to the MSC, nephrospheres and the stimulators ("third party MLR"). In both a) and b) the first column represents baseline proliferation (thymidine uptake) of activated T cells. The second column represents T cell proliferation in the presence of bone marrow-derived mesenchymal stem cells from a C57BL/6 background. These were immunosuppressive both in a 'donor derived' (a) and 'third party' (b) situation. The third and forth columns display T cell proliferation in the presence of nephrospheres isolated from an inbred C57BL/6 or outbred CD1 background respectively. Each column represents the average of nine separate wells with each well plated with cells from one biological sample representing each condition. Each measurement is corrected for a control well with the responder and modulator cells alone without the stimulators. Error bars represent standard error of the mean. Statistics calculated using one way analysis of variance with post hoc Dunnett's test (comparing all samples to controls). ** $p<0.01$; *$p<0.05$.

The lack of nephrosphere potential in adult kidney may reflect the absence of cap mesenchyme or the requirement for an altered process of enzymatic and mechanical digestion for postnatal tissue.

While nephrospheres expressed many markers suggesting a MM or CM origin, they failed to show epithelial potential in response to conventional inductive signals able to initiate renal vesicle formation *in vivo*. To address the origin of nephrospheres, E12.5 kidneys from HoxB7-GFP mice ((Srinivas et al., 1999); UB is GFP$^+$) were used to derive spheres. GFP-labelled cells formed hollow GFP$^+$ cystic structures within the first days of culture (Fig. 5C), but these did not survive initial passaging, suggesting that the ureteric epithelium cannot form nephrospheres. To investigate this further, we used Sall1-GFP transgenic mice (Takasato et al., 2004). Sall1 is a transcription factor expressed in the CM and nephrogenic interstitium as well as the early renal tubules (Takasato et al., 2004), but not expressed in other regions of the MM giving rise to interstitial elements. Cells from 30 pooled Sall1-GFP E12.5 embryonic kidneys were sorted into GFP$^+$ and GFP$^-$ populations using FACS. Approximately 40% of cells were GFP$^+$ (Fig. 5D). When cultured separately, nephrospheres were observed in all cultures, although the rate of initial cell proliferation in both



populations was reduced compared to isolations from total embryonic kidney, suggesting either FACS-related trauma or a requirement for interactions between Sall1$^+$ and Sall1$^-$ cells for optimal cell turnover (Fig. 5E). Of note, nephrospheres derived from GFP$^+$ cells ceased to show green fluorescence in culture. However, RT-PCR confirmed continued Sall1 expression in these cultures. Indeed, Sall1 expression also appeared to be present in spheres derived from initially GFP-negative sorted populations (see Fig. 5F). This suggests either nephrospheres from all sources express Sall1, but at levels lower than that required for visual detection of GFP, or that there is a selective silencing of the Sall1-GFP transgene during nephrosphere derivation despite ongoing endogenous Sall1 gene expression. In conclusion, cells able to form nephrospheres appear to be present within both the Sall1$^+$ and Sall1$^-$ population, implying their derivation from a more multipotent MM population.

Discussion

In this study, we have defined a protocol for the isolation and expansion of an embryonic renal progenitor/stem cell population with broad mesodermal potential. Using a defined serum-free media including bFGF and thrombin, we were able to culture long term self-renewing, clonal nephrospheres. These results represent the first definitive demonstration of a clonal long term repopulating population from the embryonic kidney. Previous reports have described sphere formation by cells isolated from the adult kidney, however the presence of a non-adherent sphere of cells does not signify the isolation of a stem cell without further evidence of clonality and long term self renewal data, which is absent from all previous reports (Oliver et al., 2004; Dekel et al., 2006; Gupta et al., 2006).

As discussed, there are two known embryonic populations in the kidney that have been regarded as progenitors; the metanephric mesenchyme and the cap mesenchyme, with the latter derived from the former. In order to determine which population gave rise to the cells in nephrospheres, the Sall1-positive and negative populations were tested for their nephrosphere forming potential. Sall1 is expressed both in the CM, the cortical interstitium of the nephrogenic zone and in the early nephrons. The GFP^{hi} population derived from Sall1-GFP mice has previously been shown to possess epithelial progenitor capacity (Osafune et al., 2006; Takasato et al., 2004). The fact that spheres could be cultured from both populations suggests an MM origin, including MM-derived structures not expressing Sall1. The fact that nephrospheres did not show an ability to form epithelium, except on rare occasions in reaggregation assays, supports this conclusion but also implies that when isolated the MM no longer can commit to a CM fate. Alternatively, there may exist within any nephrosphere only a very small number of cells that retain epithelial potential with the majority of the sphere being comprised of a mesodermal population with less epithelial potential.

The absence of Pax2 expression in nephrospheres, despite the positive expression of other key renal transcription factors, would also support an MM rather than a CM origin for nephrospheres. Mice null for Pax2 still develop a morphologically distinct MM, however this MM is not competent to undergo mesenchymal to epithelial transition in response to induction by the UB or other wild-type inducers (Torres et al., 1995; Brophy et al., 2001). Recent unpublished studies also suggest that if Pax2 expression is blocked in the cap mesenchyme that this tissue will convert to a more stromal phenotype (Akio Kobayashi, personal communication). Again this raises the possibility that nephrospheres arise from CM and dedifferentiate into a Pax2 negative MM-like population. This conclusion is dependent upon Pax2 expression commencing as the MM commits to CM. There is some controversy about the timing of onset of Pax2 during kidney development. In the Danforth's *short tail* mutant mouse, a defect in posterior mesoderm patterning results in a lack of branching by the ureter and failure to induce the adjacent MM. In these mice, there is an absence of Pax2 expression in the MM suggesting that the gene is expressed after induction by the UB (Phelps and Dressler, 1993). In contrast, in the c-Ret receptor knockout mouse (c-Ret expressed in UB tips), where

Figure 3 *In vitro* and *in vivo* multipotentiality of nephrospheres. A-H) Assessment of mesodermal potential *in vitro*; A,B) Nephrospheres differentiated into adipocytes in a serum-containing media supplemented with dexamethasone and insulin, as evidenced by their characteristic morphology with numerous lipid droplets that stained with oil-red-O; C,D) Differentiation of cells from nephrospheres into a myogenic phenotype was achieved by co-culture with the myoblast cell line C2C12. GFP labelled nephrospheres were dissociated and co-cultured with the C2C12 cell line. Elongated GFP labelled tubules were visible throughout the culture after 2 weeks. E,F) Osteocyte differentiation was achieved by culturing nephrospheres for three weeks in serum-containing media including ascorbic acid, β-glycerophosphate and dexamethasone. Osteocyte differentiation was achieved, as indicated by positive staining for alkaline phosphatise. Calcium deposition was detected using alizarin red S staining. Mineralization was seen in nodules versus the entire monolayer, as is traditionally seen after osteogenic differentiation of MSC (Gritti et al., 1996). G,H) Chondrocytic differentiation potential was assessed by pelleting nephrospheres in serum-free media supplemented with TGFβ3. After three weeks, the micropellet stained positive for Alcian Blue, marking cartilaginous proteoglycans. The morphology was characteristic of developing cartilage with acellular regions and nuclei residing in lacunae (arrow heads in Fig. 3H). Scale bars A-H: 50 μm. I-L) Multipotency *in vivo* was assessed via the injection of nephrosphere cells under the renal capsule. Histological analysis was assessed after three weeks. I) Low resolution image of the kidney showing the location of injected cells under the renal capsule; J) Formation of adipose under the capsule; K) evidence for early cartilage differentiation; L) Fibroblastic tissue. Scale bars I-L: 50 μm. M-P) Nephrospheres seeded in Matrigel supplemented with growth factors bFGF, EGF and HGF. ; M) Brightfield analysis of nephrospheres seeded as undissociated spheres; N-O) Immunofluorescence of such branched structures using phalloidin (red) and DAPI (blue); P) Transverse thin section of such structures stained with Toluidine blue reveals a lack of lumen. Scale bars represent 100 μm (M,N) or 20 μm (O,P).

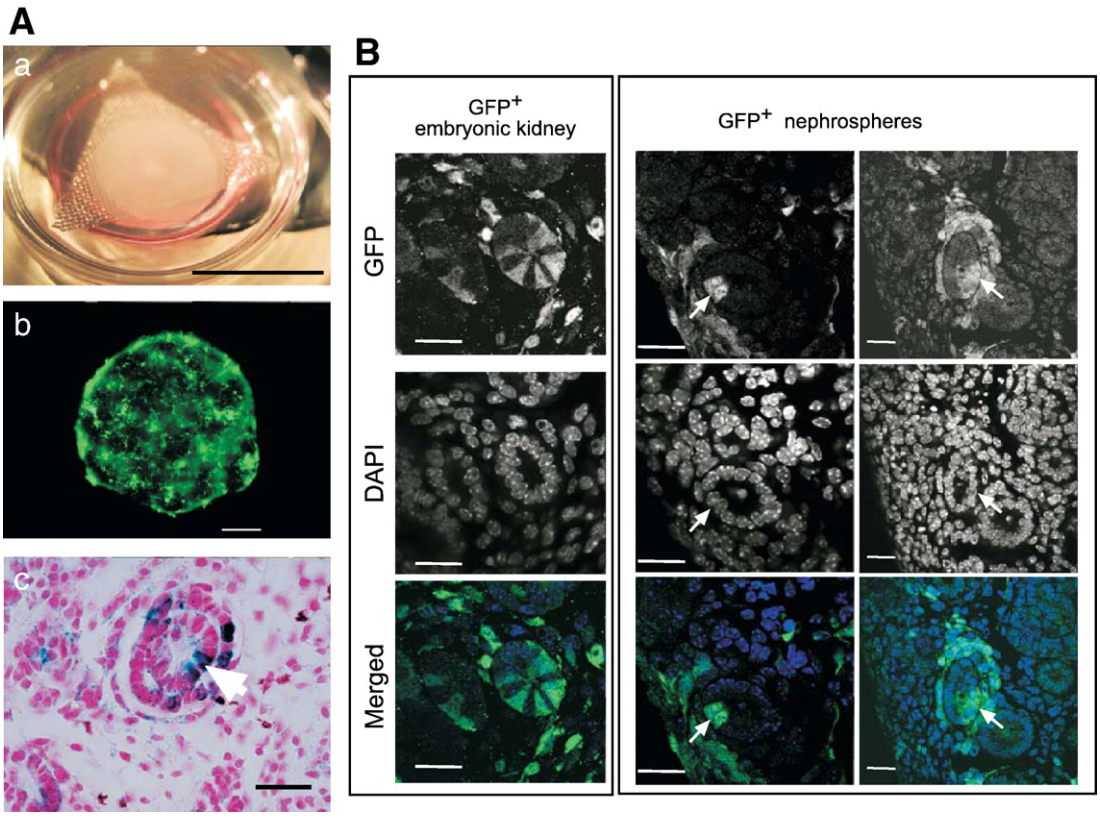


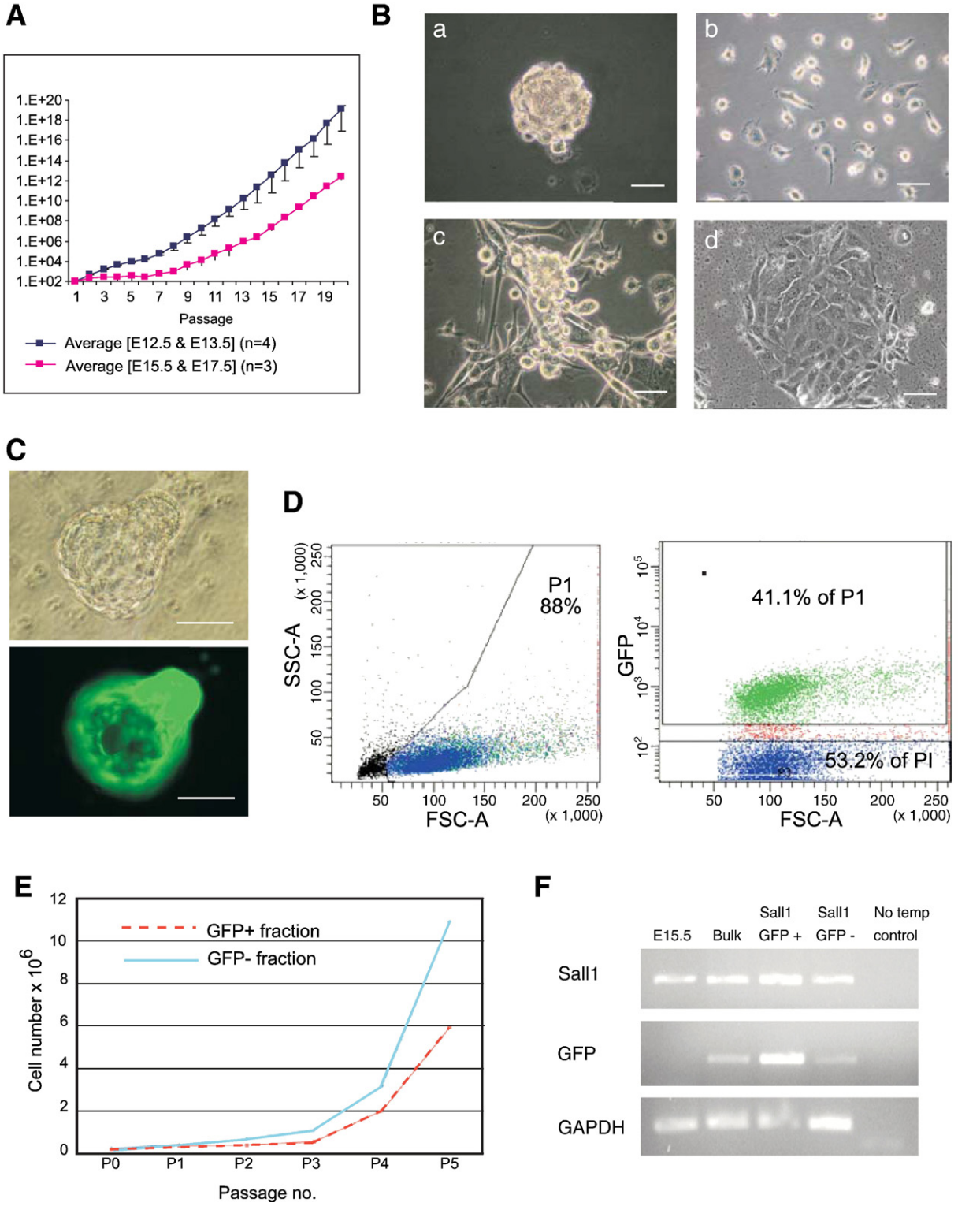
Figure 4 Assessment of renal potential of nephrospheres. A. Recombination assay in which E12.5 embryonic kidney dissociated to single cells is recombined and pelleted with test cells (another embryonic kidney, isolated cap mesenchyme of nephrosphere cells) to assess the ability of the test cells to integrate into tubular structures. a) Pelleted recombination on floating membrane in air/media culture; b) Immunofluorescence of cultured recombination between embryonic kidney and GFP-labelled nephrosphere cells. c) Section through 4 day recombination of lacZ-positive and negative wild type embryonic kidneys showing lacZ positivity within a developing nephron. This represents an example of successful renal potential of the integrating cell. B. Embryonic kidney recombinations with either constitutionally GFP+ wildtype embryonic kidney (left panels) or nephrospheres derived and cultured from the same constitutionally GFP+ embryonic kidneys. GFP indicates the location of the test cells and DAPI indicates the nuclei or all cells present. Arrows show examples of integration of nephrosphere cells into tubular structures. Scale bars represent 1 cm (Aa), 500 μm (Ab), 50 μm (Ac) and 20 μm (B).

the mesonephric duct does not form a ureteric bud, the MM is still positive for Pax2 (Brophy et al., 2001).

The genes whose expression in nephrospheres is paradoxical include Wnt4 and Flk1. In the embryonic kidney, Wnt4 is first expressed in developing renal vesicles, a population that has already undergone MET. Wnt4 null mice show condensation of mesenchyme around the UB but a reduction in the capacity to form epithelia with limited renal vesicle formation (Stark et al., 1994). The Wnt4 expression observed by RT-PCR may represent expression in only a subset of cells, given the heterogeneous nature of the nephrospheres. Alternatively, Wnt4 alone may not have been sufficient to induce this epithelial differentiation under these conditions. However, an analysis of Wnt4 expression in the developing kidney suggests that this expression is not confined to the renal vesicles but is also seen in the medullary interstitium of the developing kidney (see www.gudmap.org). Flk1 expression is commonly associated with endothelial development.

Figure 5 Determining the origin and temporal window for nephrosphere isolation. A) Comparative growth curve of nephrospheres isolated from either E12.5 - E13.5 or E15.5-17.5 embryonic kidneys. B) cultures derived from adult kidney; a-c) primary cultures from dissociated adult kidney displayed a variety of morphologies, including the formation of spheres (a), single adherent phase bright cells (b) and fibroblastic colonies (c). None of these colonies were able to be successfully passaged; d) primary cultures of proximal tubular cells isolated from adult kidney and cultured in media that retained their epithelial character, providing a comparison to what was observed from culture of adult kidney in sphere media. Scale bars: 50 μm. C) Brightfield and fluorescence image of GFP^+ cystic structure derived from Hoxb7-GFP embryonic kidney cultures in nephrosphere medium. Such structures did not survive initial passaging. Scale bars: 50 μm. D) FACS sort from Sall1-GFP mice reveals 41.1% of the viable fraction is GFP^{hi} while 53.2% is GFP-negative, E) Growth curves for nephrospheres derived from the GFP^+ and GFP- population over 5 passages; F) Analysis of GFP and Sall1 expression in nephrospheres derived from bulk embryonic kidney isolations versus GFP^+ and GFP^- fractions after 3 passages in culture.

However, Flk1 expression has also been associated with the MM both *in vivo* and *in vitro*. Flk1 expression has been reported in the CM based on immunofluorescence of embryonic kidneys and in the MM based on expression in MM-derived cell lines (Oliver et al., 2002; Usui et al., 2006). This is consistent with the hypothesis that the MM can give rise to endothelia as well as epithelia (Oliver et al., 2002). Such duality in differentiation capacity has also been shown with cells isolated from the adult renal interstitium. Such cells can give rise to cells of epithelial or endothelial character depending on conditions (Bussolati et al., 2005b). However, nephrospheres were not successfully derived from



postnatal tissue. The expression of other MM markers not associated with endothelial development and the lack of CD34 immunopositivity, a definitive marker in the mouse (Fadini et al., 2007), suggests that the spheres do not represent endothelium.

While nephrospheres appear to be derived from the MM and show broad mesenchymal potential, this population of stem cells appear to be completely restricted to the embryonic kidney and is lost from the postnatal kidney. Nephron progenitors have been proposed to exhaust around birth resulting in the cessation of nephron formation. Our observations suggest that this is also the case for the MM-derived progenitors detected using this sphere-forming assay. This would suggest that any postnatal stem cell in the kidney, should such a cell exist, is distinct from the progenitor population/s present during embryogenesis.

The requirement for thrombin and bFGF for successful nephrosphere formation is notable. Previous publications have studied the relationship between these factors in proliferation of vascular smooth muscle cells. The growth factors potentially have points of convergence in their signalling pathways (Cucina et al., 2002). Thrombin in combination with bFGF has been shown to have a synergistic effect on cell proliferation (Weiss and Nuccitelli, 1992). Thrombin also appears to mediate autocrine production of bFGF (Cucina et al., 2002) and the mitogenic effect of thrombin is dependent upon the presence of bFGF (Weiss and Maduri, 1993). Thrombin was a key factor in the development of a cardiac sphere forming assay with its addition resulting in a seven fold increase in sphere formation of cardiac stem cells. The media used was also supplemented with bFGF (Messina et al., 2004). As well as being a potent mitogen *in vitro*, thrombin is a key regulator of the injury response *in vivo*, including the coagulation pathway, inflammatory response, vessel wall healing and fibrosis (Cucina et al., 2002). Thrombin has been described as a "critical signal linking injury to regeneration" (Imokawa et al., 2004). The importance of thrombin during injury response is somewhat evolutionarily conserved, although while newt myotubes can de-differentiate and re-enter the cell cycle in response to thrombin, vertebrate myotubes are refractory to its effect (Imokawa et al., 2004). In the newt, inactivation of thrombin blocks S-phase re-entry by pigment epithelial cells of damaged newt iris (Imokawa and Brockes, 2003). Nevertheless, the link between thrombin and de-differentiation raises the possibility that the cells within a nephrosphere may have been derived from CM and have reverted to a more primitive form of intermediate mesoderm no longer competent to form nephrons as opposed to being derived from MM that had not yet committed.

During the characterisation of nephrospheres, there appeared to be a considerable degree of similarity between these cells and MSC. Immunophenotypic similarities included the expression of CD44 and CD29. Despite the wide acceptance of these as markers of MSC, these cell surface molecules are broadly expressed in a variety of tissues (De Strooper et al., 1989; Gee et al., 2004). Sca-1 positive interstitial cells from the adult kidney have also been shown to demonstrate immunosuppression (Dekel et al., 2006). More curiously, MSC expressed a number of transcription factors in common with nephrospheres and, by default, embryonic kidney. In contrast to MSC, nephrospheres did not show immune suppression capacity in a MLR. However they did show broad mesodermal potential and are αSMA positive around the edge of each sphere. Hence, the final possibility is that these cells represent an endogenous renal MSC population potentially derived from the perivascular fraction of the kidney, a region now regarded to be the source of MSC populations from most tissues (Meirelles et al., 2006). There are two strong arguments against this possibility. The first is the inability to derive such cells from postnatal tissue, from which renal MSC can be readily isolated (our own observations and 9). The second argument is the expression of specific MM genes, Wt1, Sall1 and Hoxa11, none of which are expressed in BM-MSC.

Nephrospheres could not undergo epithelialisation in vitro. This may reflect the phenotype of the sphere-initiating stem cell and/or the progeny that make up the bulk of the sphere. Alternatively, this lack of or loss of epithelial competence may have arisen during tissue culture. Pax2 and Wt1 are both critical in enabling MET (Kreidberg et al., 1993). While a morphologically distinct MM is still formed in both Pax2 and Wt1 null embryos, this MM is unresponsive to epithelial differentiation by wild-type heterologous inducers such as spinal cord (Brophy et al., 2001; Kreidberg et al., 1993). Knockdown of Pax2 in the mesenchyme but not the UB of E11.5 kidney explants also results in a failure of the mesenchyme to aggregate and undergo MET (Rothenpieler and Dressler, 1993). Valerius et al. (2002) found that the MK3 cell line, derived from embryonic kidney and believed to represent MM, could promote development and branching of isolated ureteric buds, but these cells did not undergo mesenchymal to epithelial transition and appeared "developmentally frozen". Of note, MK3 does not express Pax2 or Wt1. Hence, the lack of Pax2 expression by nephrospheres may explain the lack of epithelial potential. While Pax2 expression is necessary for MET during kidney development, Six2 and Osr1 have been shown to act as negative regulators of nephron formation. Both genes are expressed in the MM and then the CM (Self et al., 2006; James et al., 2006). Six2 maintains the CM population but prevents tubulogenesis while Osr1 is proposed to maintain embryonic renal progenitors in an undifferentiated state with overexpression in chick embryos inhibiting nephric tubule formation (James et al., 2006). It may be speculated that the expression of these two genes in the absence of Pax2 could be preventing their epithelial differentiation, thereby maintaining an undifferentiated mesodermal state.

Wilms' tumour is an embryonic neoplasia that occurs usually sporadically in children under 3 years of age (Little, 2005). The histology of the tumour is classically triphasic, including areas of blastema, stroma and attempts at epithelialisation. 10% of sporadic Wilms' tumours arise due to mutations in the WT1 gene and such tumours are proposed to represent disruptions to the normal process of differentiation of the MM – a case of arrested development (Little, 2005). In addition, the genes expressed in the blastemal components of such tumours overlaps considerably with that of the MM of the developing kidney (Metsuyanim et al., 2008). Pode-Shakked et al. (2009) have investigated the possibility that Wilms' tumours contain a cancer stem cell population by culturing cells from primary Wilms' tumour using conditions selected to promote low adherence (ultra-low attachment plates, DMEM/20%FCS/bFGF/EGF/SCF). This

did yield sphere-like aggregates, although these were not clonally passaged. The Wilms' tumour cultured populations expressed the early blastemal marker cadherin 11, showed variable levels of expression of MSC markers (CD105, CD44, CD90), haematopoietic stem cell markers (CD133, c-kit, CD34) and gene previously described as being selectively upregulated in xenografts derived from Wilms' tumours (NCAM, Fzd2, Fzd7, ACVRIIB) (Metsuyanim et al., 2008), but were negative for CD45. Individual NCAM+ cells were able to grow colonies in culture, suggesting clonogenicity, but long term clongenicity and self renewal was not proven. Some similarities exist between these putatitive Wilms' tumour cancer stem cells and the embryonic kidney nephrospheres described here, although many genes were not examined in nephrospheres. Of note, nephrosheres did not express CD34 whereas these populations did. Expression of NCAM in the developing kidney is seen in the MM, including both the CM and surrounding stroma within the neprhogenic zone. This pattern of expression is very similar to that for the Sall1 gene, supporting the concept of persistence of the embryonic nephrosphere-forming population in cases of Wilms' tumour.

As well as the classical triphasic histology seen in Wilms' tumour, these neoplasms commonly contain heterologous teratomatous regions of cartilage, bone and skeletal muscle, suggesting that at least a subcomponent of these tumours shows broad mesodermal potential similar to that of the intermediate mesoderm. In this study, we have been able to isolate a long-term, self renewing progenitor population from the embryonic kidney which shows similar broad mesodermal potential. This population is likely to represent uncommitted MM with stromal and cap mesenchyme potential, but does not appear to persist after the cessation of nephrogenesis. This suggests that it is not only the cap mesenchyme that is exhausted at the end of nephron formation but also any remaining uncommitted MM. It is of interest that such a population, were it able to persist after birth, would be able to give rise to the many different cell types observed in Wilms' tumor. As our data suggests that this population should not persist, this may explain why Wilms' tumour is restricted to early life and may explain in part why adult Wilms' tumour is a very rare phenomenon (Kartsanis et al., 2007).

Acknowledgments

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Appendix A. Supplementary data

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References

Appel, D., Kershaw, D., Smeets, B., Yuan, G., Fuss, A., Frye, B., Elger, M., Kriz, W., Floege, J., Moeller, M.J., 2009. Derivation of podocytes from the parietal cell compartment. J. Am. Soc. Nephrol. 20 (2), 333–343.

Bartholomew, A., Sturgeon, C., Siatskas, M., Ferrer, K., McIntosh, K., Patil, S., Hardy, W., Devine, S., Ucker, D., Deans, R., Moseley, A., Hoffman, R., 2002. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp. Hematol. 30, 42–48.

Bearzi, C., Rota, M., Hosoda, T., Tillmanns, J., Nascimbene, A., De Angelis, A., Yasuzawa-Amano, S., Trofimova, I., Siggins, R.W., Lecapitaine, N., Cascapera, S., Beltrami, A.P., D'Alessandro, D.A., Zias, E., Quaini, F., Urbanek, K., Michler, R.E., Bolli, R., Kajstura, J., Leri, A., Anversa, A.P., 2007. Human cardiac stem cells. Proc. Natl. Acad. Sci. U.S.A. 104, 14068–14073.

Bowes III, R.C., Lightfoot, R.T., van de Water, B., Stevens, J.L., 1999. Hepatocyte growth factor induces tubulogenesis of primary renal proximal tubular epithelial cells. J. Cell. Physiol. 180, 81–90.

Brophy, P.D., Ostrom, L., Lang, K.M., Dressler, G.R., 2001. Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. Development 128, 4747–4756.

Bussolati, B., Bruno, S., Grange, C., Buttiglieri, S., Deregibus, M.C., Cantino, D., Camussi, M., Isolation, M., 2005a. of renal progenitor cells from adult human kidney. Am. J. Pathol. 166, 545–555.

Bussolati, B., Bruno, S., Grange, C., Buttiglieri, S., Deregibus, M.C., Cantino, D., Camussi, G., 2005b. Isolation of renal progenitor cells from adult human kidney. Am. J. Pathol. 166, 545–555.

Campos, L.S., Leone, D.P., Relvas, J.B., Brakebusch, C., Fassler, R., Suter, U., French-Constant, C., 2004. Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. Development 131, 3433–3444.

Carroll, T.J., Park, J.S., Hayashi, S., Majumdar, A., McMahon, A.P., 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev. Cell. 9 (2), 283–292.

Challen, G.A., Bertoncello, I., Deane, J., Ricardo, S., Little, M.H., 2006. Kidney side population cells represent a non-haematopoietic but heterogeneous population with multilineage and renal potential. J. Am. Soc. Nephrol. 17 (7), 1896–1912.

Coresh, J., Selvin, E., Stevens, L.A., Manzi, J., Kusek, J.W., Eggers, P., et al., 2007. Prevalence of chronic kidney disease in the United States. JAMA 298, 2038–2047.

Cucina, A., Borrelli, V., Lucarelli, M., Sterpetti, A.V., Cavallaro, A., Strom, R., Santoro-D'Angelo, L., Scarpa, S., 2002. Autocrine production of basic fibroblast growth factor translated from novel synthesized mRNA mediates thrombin-induced mitogenesis in smooth muscle cells. Cell Biochem. Funct. 20, 39–46.

De Strooper, B., Van der Schueren, B., Jaspers, M., Saison, M., Spaepen, M., Van Leuven, F., Van den Berghe, H., Cassiman, J.J., 1989. Distribution of the beta 1 subgroup of the integrins in human cells and tissues. J. Histochem. Cytochem. 37, 299–307.

Dekel, B., Zangi, L., Shezen, E., Reich-Zeliger, S., Eventov-Friedman, S., Katchman, H., Jacob-Hirsch, J., Amariglio, N., Rechavi, G., Margalit, R., Reisner, Y., 2006. Isolation and characterization of nontubular sca-1+lin- multipotent stem/progenitor cells from adult mouse kidney. J. Am. Soc. Nephrol. 17, 3300–3314.

Di Rocco, G., Iachininoto, M.G., Tritarelli, A., Straino, S., Zacheo, A., Germani, A., Crea, F., 2006. Capogrossi MC.Myogenic potential of adipose-tissue-derived cells. J. Cell Sci. 119, 2945–2952.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.S., Wicha, M.S., 2003. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. 17, 1253–1270.

Fadini, G.P., Avogaro, A., Agostini, C., 2007. Critical assessment of putative endothelial progenitor phenotypes. Exp. Hematol. 35, 1479–1480.

Gee, K., Kryworuchko, M., Kumar, A., 2004. Recent advances in the regulation of CD44 expression and its role in inflammation and autoimmune diseases. Arch. Immunol. Ther. Exp. (Warsz) 52, 13–26.

Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D.J., Roisen, F., Nickel, D.D., Vescovi, A.L., 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J. Neurosci. 16, 1091–1100.

Gupta, S., Verfaillie, C., Chmielewski, D., Kren, S., Eidman, K., Connaire, J., Heremans, Y., Lund, T., Blackstad, M., Jiang, Y., Luttun, A., Rosenberg, M.E., 2006. Isolation and characterization of kidney-derived stem cells. J. Am. Soc. Nephrol. 17, 3028–3040.

Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M., Nagy, A., 1998. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech. Dev. 76, 79–90.

Han, H.J., Sigurdson, W.J., Nickerson, P.A., Taub, M., 2004. Both mitogen activated protein kinase and the mammalian target of rapamycin modulate the development of functional renal proximal tubules in matrigel. J. Cell Sci. 117, 1821–1833.

Haniffa, M.A., Wang, X.N., Holtick, U., Rae, M., Isaacs, J.D., Dickinson, A.M., Hilkens, C.M., Collin, M.P., 2007. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J. Immunol. 179, 1595–1604.

Hartman, H.A., Lai, H.L., Patterson, L.T., 2007. Cessation of renal morphogenesis in mice. Dev. Biol. 310 (2), 379–387.

Hopkins, C., Li, J., Rae, F., Little, M.H., 2009. Stem cell options for kidney disease. J. Pathol. 217 (2), 265–281.

Humphreys, B.D., Valerius, M.T., Kobayashi, A., Mugford, J.W., Soeung, S., Duffield, J.S., et al., 2008. Intrinsic epithelial cells repair the kidney after injury. Cell Stem Cell 2, 284–291.

Imokawa, Y., Brockes, J.P., 2003. Selective activation of thrombin is a critical determinant for vertebrate lens regeneration. Curr. Biol. 13, 877–881.

Imokawa, Y., Simon, A., Brockes, J.P., 2004. A critical role for thrombin in vertebrate lens regeneration. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 765–776.

James, R.G., Kamei, C.N., Wang, Q., Jiang, R., Schultheiss, T.M., 2006. Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells. Development 133, 2995–3004.

Jessberger, S., Clemenson Jr., G.D., Gage, F.H., 2007. Spontaneous fusion and nonclonal growth of adult neural stem cells. Stem Cells 25, 871–874.

Kartsanis, G., Douros, K., Ravazoula, P., Fokaefs, E., 2007. Adult Wilms' tumor: a case report and review of literature. Int. Urol. Nephrol. 39 (1), 3–6.

Kispert, A., Vainio, S., McMahon, A.P., 1998. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. Development 125, 4225–4234.

Kitamura, S., Yamasaki, Y., Kinomura, M., Sugaya, T., Sugiyama, H., Maeshima, Y., Makino, H., 2005. Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. Faseb J. 19, 1789–1797.

Kobayashi, A., Valerius, M.T., Mugford, J.W., Carroll, T.J., Self, M., Oliver, G., McMahon, A.P., 2008. Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. Cell Stem Cell. 3 (2), 169–181.

Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., Jaenisch, R., 1993. WT-1 is required for early kidney development. Cell 74, 679–691.

Lawson, D.A., Xin, L., Lukacs, R.U., Cheng, D., Witte, O.N., 2007. Isolation and functional characterization of murine prostate stem cells. Proc. Natl. Acad. Sci. U. S. A. 104, 181–186.

Little, M.H., 2005. Starting the kidney over again. Developmental Biology of Neoplastic Growth. : Prog. Mol. Subcell Biol., vol. 40. Springer-Verlag, pp. 107–132.

Maeshima, A., Yamashita, S., Nojima, Y., 2003. Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney. J. Am. Soc. Nephrol. 14, 3138–3146.

Maeshima, A., Sakurai, H., Nigam, S., 2006. Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney. J. Am. Soc. Nephrol. 17, 188–198.

Meirelles, L., Chagastelles, P.C., Nardi, N.B., 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J. Cell Sci. 119, 2204–2213.

Messier, B., Leblond, C.P., 1960. Cell proliferation and migration as revealed by autoradiography after injection of thymidine-H^3 into male rats and mice. Am. J. Anat. 106, 247–285.

Messina, E., De Angelis, L., Frati, G., Morrone, S., Chimenti, S., Fiordaliso, F., Salio, M., Battaglia, M., Latronico, M.V., Coletta, M., Vivarelli, E., Frati, L., Cossu, G., Giacomello, A., 2004. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ. Res. 95, 911–921.

Metsuyanim, S., Pode-Shakked, N., Schmidt-Ott, K.M., Keshet, G., Rechavi, G., Blumental, D., Dekel, B., 2008. Accumulation of malignant renal stem cells is associated with epigenetic changes in normal renal progenitor genes. Stem Cells 26 (7), 1808–1817.

Murphy, A.J., Viero, S., Ho, M., Thorner, P.S., 2009. Diagnostic utility of nestin expression in pediatric tumors in the region of the kidney. Appl. Immunohistochem. Mol. Morphol. 17 (6), 517–523.

Oliver, J.A., Barasch, J., Yang, J., Herzlinger, D., 2002. D., Q. Al-Awqati. Metanephric mesenchyme contains embryonic renal stem cells. Am. J. Physiol. Renal. Physiol. 283, F799–F809.

Oliver, J., Maarouf, O., Cheema, F.H., Martens, T.P., Al-Awqati, Q., 2004. The renal papilla is a niche for adult kidney stem cells. J. Clin. Invest. 114, 795–804.

Osafune, K., Takasato, M., Kispert, A., Asashima, M., Nishinakamura, R., 2006. Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay. Development 133, 151–161.

Phelps, D.E., Dressler, G.R., 1993. Aberrant expression of Pax-2 in Danforth's short tail (Sd) mice. Dev. Biol. 157, 251–258.

Pode-Shakked, N., Metsuyanim, S., Rom-Gross, E., Mor, Y., Fridman, E., Goldstein, I., Amariglio, N., Rechavi, G., Keshet, G., Dekel, B., 2009. Developmental tumourigenesis: NCAM as a putative marker for the malignant renal stem/progenitor cell population. J. Cell. Mol. Med. 13 (8B), 1792–1808.

Reynolds, B.A., Rietze, R.L., 2005. Neural stem cells and neurospheres–re-evaluating the relationship. Nat. Meth. 2, 333–336.

Reynolds, B.A., Weiss, S., 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255, 1707–1710.

Robertson, N.J., 2007. P.J., Fairchild, H. Waldmann. Ectopic transplantation of tissues under the kidney capsule. Methods Mol. Biol. 380, 347–354.

Ronconi, E., Sagrinati, C., Angelotti, M.L., Lazzeri, E., Mazzinghi, B., Ballerini, L., Parente, E., Becherucci, F., Gacci, M., Carini, M., Maggi, E., Serio, M., Vannelli, G.B., Lasagni, L., Romagnani, S., Romagnani, P., 2009. Regeneration of glomerular podocytes by human renal progenitors. J. Am. Soc. Nephrol. 20 (2), 322–332.

Rothenpieler, U.W., Dressler, G.R., 1993. Pax-2 is required for mesenchyme-to-epithelium conversion during kidney development. Development 119, 711–720.

Santos, O.F., Nigam, S.K., HGF-induced, S.K., 1993. tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF-beta. Dev. Biol. 160, 293–302.

Self, M., Lagutin, O.V., Bowling, B., Hendrix, J., Cai, Y., Dressler, G.R., Oliver, G., 2006. Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. EMBO J. 25, 5214–5228.

Short, B.J., Brouard, N., Simmons, P.J., 2009. Prospective isolation of mesenchymal stem cells from mouse compact bone. Methods Mol. Biol. 482, 259–268.

Singec, I., Knoth, R., Meyer, R.P., Maciaczyk, J., Volk, B., Nikkhah, G., Frotscher, M., Snyder, E.Y., 2006. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat. Meth. 3, 801–806.

Srinivas, S., Goldberg, M.R., Watanabe, T., D'Agati, V., al-Awqati, Q., Costantini, F., 1999. Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. Dev. Genet. 24 (3-4), 241–251.

Stark, K., Vainio, S., Vassileva, G., McMahon, A.P., 1994. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372, 679–683.

Takasato, M., Osafune, K., Matsumoto, Y., Kataoka, Y., Yoshida, N., Meguro, H., Aburatani, H., Asashima, M., Nishinakamura, R., 2004. Identification of kidney mesenchymal genes by a combination of microarray analysis and Sall1-GFP knockin mice. Mech. Dev. 121, 547–557.

Taub, M., Wang, Y., Szczesny, T.M., Kleinman, H.K., 1990. Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in matrigel cultures in serum-free medium. Proc. Natl Acad. Sci. U. S. A. 87, 4002–4006.

Toma, T.G., McKenzie, I.A., Bagli, D., Miller, F.D., 2005. Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells 23, 727–737.

Torres, M., Gomez-Pardo, E., Dressler, G.R., Gruss, P., 1995. P. Pax-2 controls multiple steps of urogenital development. Development 121, 4057–4065.

Turner, B.E., Kambouris, M.E., Sinfield, L., Lange, J., Burns, A.M., Lourie, R., Atkinson, K., Hart, D.N., Munster, D.J., Rice, A.M., 2008. Reduced intensity conditioning for allogeneic hematopoietic stem-cell transplant determines the kinetics of acute graft-versus-host disease. Transplantation 86 (7), 968–976.

Usui, J., Yamada, R., Kanemoto, K., Koyama, A., Nagata, M., 2006. Murine metanephric mesenchyme possesses characteristics of vascular endothelial cells in vitro. Nephron. Exp. Nephrol. 102, e93–e98.

Valerius, M.T., Patterson, L.T., Witte, D.P., Potter, S.S., 2002. Microarray analysis of novel cell lines representing two stages of metanephric mesenchyme differentiation. Mech. Dev. 112, 219–232.

Wachs, F.P., Couillard-Despres, S., Engelhardt, M., Wilhelm, D., Ploetz, S., Vroemen, M., Kaesbauer, J., Uyanik, G., Klucken, J., Karl, C., Tebbing, J., Svendsen, C., Weidner, N., Kuhn, H.G., Winkler, J., Aigner, L., 2003. High efficacy of clonal growth and expansion of adult neural stem cells. Lab. Invest. 83, 949–962.

Weiss, R.H., Maduri, M., 1993. The mitogenic effect of thrombin in vascular smooth muscle cells is largely due to basic fibroblast growth factor. J. Biol. Chem. 268, 5724–5727.

Weiss, R.H., Nuccitelli, R., 1992. Inhibition of tyrosine phosphorylation prevents thrombin-induced mitogenesis, but not intracellular free calcium release, in vascular smooth muscle cells. J. Biol. Chem. 267, 5608–5613.